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The Value of the Cytokinome Profile

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1. Introduction

Many scientific articles describe the pathogenesis of diseases that afflict the modern man (cancer, diabetes, obesity, degenerative diseases, etc.) as a slow common inflammatory process that is the basis of all these diseases. Therefore, we commonly speak of chronic inflammatory diseases (Allavena et al., 2008). The basis of this statement are the numerous experimental observations which show that these diseases are driven, from the earliest moments, by exchange between cells of tissues and organs of molecules that operate as messengers. These molecules, carrying biological messages of great importance, inform and lead a complex system of different cell types on what happens and towards which physiological and metabolic changes they are being carried. The chemical nature of these signaling molecules is diverse, but a group of them, the cytokines, is among the most important and studied inter-cellular messengers (Germano et al., 2008). We know the biological meaning of the signal of many of them, thus we can generally divide these molecules into two major classes: pro-inflammatory cytokines and anti-inflammatory cytokines. They are small proteins, quite numerous, more than about 100, expressed in very low amounts (pico and nano molar) and often short-lived, to cover specific information needs (Macarthur et al., 2004).

The cells recognize these signals through appropriate receptors placed on their external membranes. However their study had some limitations due to the fact that (i) only those more abundant were studied, even if with very sensitive assays based on use of antibodies and fluorescence (ELISA); (ii) the receptors show pleiotropy, i.e. they have good affinity for various cytokines and hence the message can be brought by different cytokines; (iii) the biological significance of the message is known only for some of them, for example, it is not known which is the biological meaning carried out by the under-represented cytokines (the less concentrated ones at phenotypic level) and if the different messages are recognized by the receptor as only redundant or with diverse biological content (Colvin et al., 2004; Costantini et al., 2009; Trotta et al., 2009).

Recently, specific protein chips of considerable and improved sensitivity are being developed. They allow the simultaneous determination of different cytokines based on a fluorescence/laser/antibodies technology which uses microparticle beads (multiplex technology) that allows the analysis of tiny samples (few dozens of microliter) of serum, plasma, or cell cultures supernatant. Each bead set is coated with capture antibody specific for one analyte. The result is the most accurate, sensitive, and reproducible cytokine assay available. An important point of this technology is the ability to appreciate quantitatively also the presence of the under-represented cytokines (Capone et al., 2010; Costantini et al., 2010a). The pattern of these cytokines, being part of the new global or holistic logic, which is used today in the "omics" approach to the study of biological phenomena, can be indicated as "cytokinome" (Costantini et al., 2010b).

The fact is that the cytokines form an informative network, for some ways very similar to the Internet, that capillary connects, as knots to the network, cellular systems also different. The study of this network is important for understanding the evolution of the pathogenesis of many chronic inflammatory diseases. However, there are many questions that must still find the answer. In the case of chronic inflammatory diseases, which development in the time the whole pattern of cytokines shows? Their evolution in time begins in the same way and is common for all the diseases or is pathology correlated and addressed by different types or classes of cytokines? Which one is the cytokinome development during the disease? Answers to these and other questions are essential not only to be able to describe the cytokinome dynamics during the progression of chronic inflammatory diseases but, above all, to try to predict in large advance the prognosis of the disease. If this will be possible, we will be able to intervene with great advance in the early stages of the disease with much more chance of healing or of extending the duration and the expectations of life.

Therefore, the review will focus on:

- Role of the cytokines in chronic inflammatory diseases and cancers
- Challenge and significance of the cytokinome profile
- Hepatocarcinoma as an example of chronic inflammatory disease
- Metabolic pathway analysis of significant genes in hepatoma cells
- Evaluation of cytokines in HCC patients with HCV-related cirrhosis
- Evaluation of cytokines in patients with chronic HCV or with HCV-related cirrhosis
- The need of cytokinome data mining system for a predictive medicine for chronic inflammatory diseases
- The need for structural studies of cytokine/receptor complex: the example of CXCL9, CXCL10 and CXCL11 chemokines and their membrane receptor CXCR3

2. Role of the cytokines in chronic inflammatory diseases and cancers

Inflammation is a physiologic process in response to acute tissue damage resulting from physical injury, ischemic injury, infection, exposure to toxins, chemical irritation, and/or wounding or other types of trauma (Lu et al., 2006; Philip et al., 2004); it is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissue. At the very early stage of inflammation, the phagocytic cells are mainly involved: neutrophils are the first cells to migrate to the inflammatory sites under the regulation of molecules produced by rapidly responding macrophages and mast cells prestationed in tissues (Coussens & Werb, 2002). As the inflammation progresses, various

types of leukocytes, lymphocytes, and other inflammatory cells are activated and attracted to the inflamed site by a signaling network involving a great number of growth factors, cytokines, and chemokines (Coussens & Werb, 2002). All cells recruited to the inflammatory site contribute to tissue breakdown and are beneficial by strengthening and maintaining the defense against infection (Coussens & Werb, 2002). The resolution of inflammation also requires a rapid programmed clearance of inflammatory cells: neighboring macrophages, dendritic cells, and backup phagocytes do this job by inducing apoptosis and conducting phagocytosis (Savill et al., 2002).

However, inflammation may become chronic either because an inflammatory stimulus persists or because of dysregulation in the control mechanisms that normally turn the process off. Recently, it has been suggested that inflammation associated with cancer is similar to that seen with chronic inflammation, which includes the production of growth and angiogenic factors that stimulate tissue repair, factors that can also promote cancer-cell survival, implantation, and growth (Philip et al., 2004; Macarthur et al., 2004; Balkwill and Mantovani, 2001). Interestingly, inflammation functions at all three stages of tumor development: initiation, progression and metastasis.

Since many cancers arise from sites of infection, chronic irritation, and inflammation, it is now clear that the tumor microenvironment, which is largely orchestrated by inflammatory cells and cytokines (Fig. 1), is an indispensable participant in the neoplastic process altering not only the metabolic needs of the tissue, but also fostering DNA and protein damage, proliferation, survival, mutagenesis, migration and metastasis of malignant cells (Allavena et al., 2008). Indeed all tumors in the presence of stromal and infiltrating inflammatory cells are facilitated and helped to maintain these metastatic processes. Leukocytes, lymphocytes and other inflammatory cells are activated in this process and attracted to the inflamed site. Inflammation contributes to initiation by inducing the release of a variety of pro-inflammatory cytokines and chemokines and inflammatory enzymes as cyclo-oxygenases that alert the vasculature to release inflammatory cells and factors into the tissue milieu, thereby causing oxidative damage, DNA mutations, and other changes in the microenvironment, making it more conducive to cell transformation, increased survival and proliferation (Germano et al., 2008). We must not forget that many cytokines and chemokines are inducible by hypoxia which is a major physiological difference (Mancino et al., 2008). An important aspect of the tumor microenvironment is the cytokine mediated communication between the tumor and cells. Cytokines and chemokines have many activities that permit cell–cell communication locally at the tissue, with the outcome determined by cytokine concentration milieu and cell type (Germano et al., 2008). Current thinking is that activated immune cells provide both anti- and protumorigenic signals, thus representing targets to be harnessed or attacked for therapeutic advantage depending upon environmental and/or cellular context. Because the control of cytokine production is highly complex and multifactorial, the effects of cytokines are mediated through multiple regulatory networks. The intricate complexity of both cytokine networks clearly conceals the role that a single cytokine may play in the pathogenesis of the disease. It is therefore informative to investigate the immunopathogenesis of a disease process by analyzing multiple cytokines. In this way it is possible to provide a better understanding of the role of cellular, humoral and chemotactic immunity at a critical time in some cancer diseases and also in the treatment course of a correlated infection (Costantini et al., 2009).

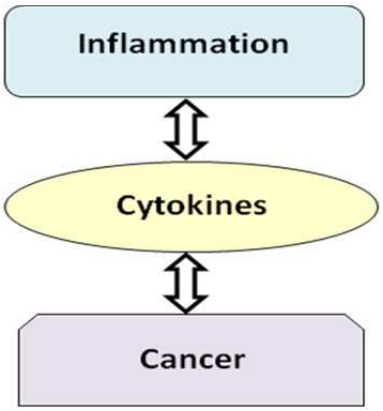

Fig. 1. Relationship between inflammation, cytokines and cancer

3. Challenge and significance of the cytokinome profile

In order to understand the whole universe of human cytokines, the socalled cytokinome, according the "omics" system of definition, it needs to evaluate these proteins and analyse their complex network of interactions by which they regulate their own synthesis or that of their receptors, and antagonize or synergize with each other in many and often redundant ways (Costantini et al., 2010b).

A major effort is the achievement of an efficient database that can collect together correct ontologies, algorithms and tools of analyses, structural and "omics" data of cytokines and their receptors, metabolic pathways, and the whole interactome. Another intriguing problem related to the cytokine family and their receptors is the pleiotropy existing in the cytokine system, where one cytokine is able to activate various receptors and many different cytokines activate the same receptor. When the frame of the whole cytokine network will be known, we will have the possibility to create best and more efficient drugs against the cancer, most probably able to interact with the receptors rather than directly with the cytokine molecules because of their pleiotropic effect. Another element of complexity in the cytokine network is introduced also by the fact that some genes encoding cytokines can give rise to variant forms of cytokines (isoforms) by means of alternative splicing, yielding molecules with slight structural differences but biologically significant changes of activities. This explains why it is always useful to analyze the gene expression profile correlated to the cytokines. In fact, previous studies have identified important mutations in some cancers, but they were primarily focused on a limited set of genes and, thus, provided a constrained view of the mutational spectrum.

However, a correct and comprehensive understanding of cytokine functions can be obtained from simultaneous and coherent measurements of the serum concentrations of cytokines. This point raises the inherent difficulty of a simultaneous measurement of the cytokine concentrations to obtain correct internal ratios among the various molecules present in the same biological fluid due to the often large difference in concentrations spanning several magnitude orders. At present, it is possible to effectively characterize the serum levels of cytokines using a broad-spectrum bead based multiplex immunoassay.

In this complex interactions network, Systems Biology and/or Biologically Integrated Approaches are powerful tools to analyze as a whole, the enormous amount of data coming from the so-called "omics" disciplines (genomics, transcriptomics, proteomics) by computational methods and algorithms, in order to create an information body that allows us to have a comprehensive and integrated vision of the biological phenomenon under investigation. In fact, until the last century, the approach of biological science was to break down the object of study in its elementary parts and to study all the singular units in order to explain the life processes. This was a typical analytical and reductionist procedure, which allowed the understanding of almost all properties of molecular parts of living organisms, such as genes, proteins, metabolites, and was focused on the study of each single component of the system under consideration but was not able to predict the behavior of the systems as a whole. A system can be defined as a number of interacting elements existing within a boundary that is surrounded by an environment. Therefore, a complex system is able to create new properties from the interactions between its components, and also to interact and to respond with the external inputs. When the interactions between the parties are determined by the dynamical processes inducing the emerging properties like adaptability, self-organization and the ability to respond under disturbance, the system becomes complex. In this way these non linear interactions allow a number of possible several states and new emergent behaviors are not predictable from the simple sum of the component parts. These principles were applied to study the living organisms, the stock markets, the ecosystems and the flock of birds. In biology it's necessary to study the living organism as a whole, and the laws of regarding the organizational forces of systems, which yet are not well known, but are essential to solve and to understand the collective phenomena and the framework for the functionality of the systems (Costantini et al., 2008).
Therefore, all the data related to the cytokine evaluations can be analyzed and modeled computationally by using graphs or networks connecting the various data groups (related to gene and protein expression obtained by microarrays and by multiplex biometric ELISA-based immunoassay) in terms of dynamic probabilistic maps of metabolic and/or physiological activities and/or pathogenetic pathways. Hence, the definition and evaluation of a human cytokinome is an important future tool to analyze the interaction network of cytokines both in healthy individuals and in patients affected from a cancer. Using these computational models it will be easier and immediate to understand and investigate how the regression of a chronic inflammation process, by acting on the cellular populations of cytokines, can block the progression of the cancer and how this knowledge can be an useful prognostic and diagnostic tool for clinicians.

4. Hepatocellular carcinoma as an example of chronic inflammatory disease

Hepatocellular carcinoma (HCC) accounts for >5% of all human cancers and for 80% - 90% of primary liver cancer. It is a major health problem worldwide being the fifth most common malignancy in men and the eighth in women; the third most common cause of cancer-related death in the world. Moreover early diagnosis is uncommom and medical treatments are inadeguate (Altekruse et al., 2009).
Yearly 550,000 people worldwide die for HCC, with a 2:1 ratio for men versus women. Its incidence is increasing dramatically, with marked variations among geographic areas (Jemal et al., 2007), racial and ethnic groups, environmental risk factors. The estimated annual number of HCC cases exceeds 700,000, with a mean annual incidence of 3-4% (Jemal et al.,

2007). Most HCC cases (>80%) occur in either sub-Saharan Africa or in Eastern Asia (China alone accounts for more than 50% of the world's cases) (Jemal et al., 2007). In the United States (US) HCC incidence is lower than other countries (0.3/100000) even if there has been a significant and alarming increase in the incidence of HCC in the US, from 1.3 in the late 70s' to 3 in the late 90s', due to HCV infection. In 2008, 21370 new cases of HCC and intrahepatic bile duct cancer were estimated with 18410 deaths (Jemal et al., 2007). In Europe, Oceania and America, chronic hepatitis C and alcoholic cirrhosis are the main risk factors for HCC. The main risk factor for HCC development in patients with hepatitis C is the presence of cirrhosis. Among patients with hepatitis C and cirrhosis, the annual incidence rate of HCC ranges between 1-8%, being higher in Japan (4-8%) intermediate in Italy (2-4%) and lower in USA (1.4%) (Fassio, 2010). Analysis of mortality from HCC in Europe confirmed large variability, with high rates in France (6.79/100000) and Italy (6.72/100000) due to hepatitis C virus (HCV) during the 1960s and 1970s (Bosetti et al., 2008). Southern Italy has the highest rates of HCC in Europe (Fusco et al., 2008).

HCC is unique among cancers occurring mostly in patients with a known risk factor: ninety percent of HCCs develop in the context of chronic liver inflammation and cirrhosis (Altekruse et al., 2009). Hepatitis B (HBV) and C (HCV) viruses are the major cause of liver disease worldwide. Fortunately, the hepatitis B virus vaccine has resulted in a substantial decline in the number of new cases of acute hepatitis B among children, adolescents, and adults in western countries since the mid-1980s. This success is not duplicable for HCV where active or passive vaccination is not available yet. Therefore, the present and next future HCC history will be mainly related to HCV infection. The incidence of HCV infection is hard to quantify since it is often asymptomatic. The World Health Organization estimates that 3% of the world's population - more than 170 million people - are chronically infected (3-4 million new infections every year). Therefore, a tremendous number of people are currently at elevated risk for HCC and its early diagnosis (when surgical intervention is possible) may significantly affect the patients prognosis (Ryder, 2003).

However it is possible also a direct carcinogenesis by hepatitis viruses, without a cirrhotic step (Nash et al., 2010). In particular, it was reported that patients without cirrhosis were younger, survived longer than patients with cirrhosis ($P < 0.0001$) and had a better 5-year survival experience (Chiesa et al., 2000).

In contrast to HBV, HCV does not integrate into the host genome and does not contain a reverse transcriptase. In particular, in the infected subjects both viruses trigger an immune-mediated inflammatory response (hepatitis) that either clears the infection or slowly destroys the liver (Bowen & Walker, 2005).

Effective HCV immunity is limited by the high variability of virion genome; HCV virions turn over rapidly (with a half-life of about 3 h), and up to about 1012 complete viruses are produced per day in an infected person (Ueno et al., 2009). About 80% of newly infected patients develop chronic infection; an estimated 10% to 20% will develop cirrhosis and 1-5% proceeds to end-stage liver cancer over a period of 20 to 30 years (Fig. 2). In the case of HCV, HCC is invariably observed as a complication of cirrhosis, whereas in the case of HBV HCC is often found in non-cirrhotic liver. Therefore, the hepatic fibrosis dramatically increase the incidence of HCC (Castello et al., 2010a).

Many studies were conducted in the last years in regard to anti-HCV immune response. In fact, much attention has recently focused on regulatory T cells (Tregs) being able to secrete inhibitory cytokines such as IL-10 or TGF-β, even if their contribution is yet unclear (Castello

et al., 2010b). Increased Treg cells were found in peripheral blood of HCV-infected patients (Boettler et al., 2005) as well as in the tumor microenvironment of HCC patients (Ormandi et al., 2005). The frequency of naturally arising CD4+CD$^{25high+}$ Tregs in the periphery of HCV-infected patients was reported to be higher than that in patients who resolved the infection or uninfected controls (Cabrera et al., 2004). TH1 cytokines are generally up-regulated in patients with HCC, resulting in higher levels of pro-inflammatory cytokines, as IL-1α, IL-15, IL-18, TNF-α, TNF-αRs, TNF-αRI, TNF-αRII, and IL-6 in comparison with healthy individuals (Huang et al., 1999). However, the intra/peri-tumoral cytokines levels are often different from the serum levels (Budhu & Wang, 2006). Higher serum IL-6 level was an independent risk factor for HCC development in female but not male chronic hepatitis C patients (Nakagawa et al., 2009). IL-10 was highly expressed in HCC tumors and serum, correlating with disease progression (Budhu & Wang, 2006). Budhu and Wang reviewed the association between cytokine abnormalities and HCC patients and found that a dominant TH2-like cytokine profile (IL-4, IL-8, IL-10, and IL-5) and a decrease in the TH1-like cytokines (IL-1α, IL-1β, IL-2, IL-12p35, IL-12p40, IL-15, TNF-α, and IFN-γ was associated with the metastatic phenotype of disease (Budhu & Wang, 2006). Thus, it has been hypothesized that TH1 cytokines are involved in tumor development, whereas TH2 cytokines in tumor progression. Recently the cytokine concentrations have been evaluated in patients with HCC patients with HCV-related cirrhosis (Capone et al., 2010).

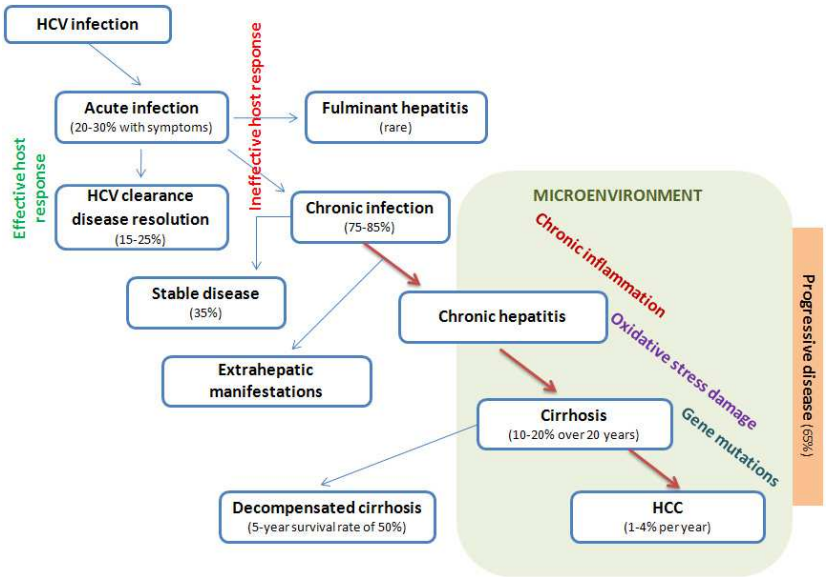

Fig. 2. Evolution from HCV infection to HCC.

5. Metabolic pathway analysis of significant genes in hepatoma cells

The cytokinome study is an important step to our understanding of chronic inflammatory diseases because a global and dynamic knowledge of cellular signaling, at moment only static and mechanistic, will improve our ability to adequately read and understand the

meaning in the early time of the information that cells exchange each other. The present technology supports this view from an experimental point of view by DNA and Protein microarrays. These techniques taken together can support a better knowledge of the relationships existing between genome behavior and related phenotypes (cytokines in this case) during the progression of a disease. More difficult is to correlate genomic and phenotypic levels by a logical analysis able to correlate the genes action with their products to extract useful biological information on the disease progression, such as changes in metabolic pathways or activation of new metabolic paths. In general, the knowledge of early metabolic changes during the first stages of an illness is an important moment to develop new more specific drugs by pharmacogenomics as well as to operate a metabolic repair by nutrigenomics. Probably this holistic view of the medicine is perhaps more expensive for the community but it is necessary to efficiently fight the numerous diseases of our time unfortunately founded on chronic inflammation.

The global gene expression has been evaluated in HepG2 cells in comparison to normal human hepatocytes using Illumina microarray. In particular, cRNAs were hybridized to the HumanWG-6 Bead-Chip array which allows to assess the presence of more than 48,000 transcripts (Whole Genome). Our metabolic pathway analysis aims at discovering modifications in and/or activation of new metabolic pathways involved into a perturbation of the hepatoma cell homeostasis. We have used a cluster analysis or "clustering" that is the assignment of a set of data or observations into subsets (called clusters) so that observations in the same cluster are similar. This is a method of analysis used also in bioinformatics to evaluate high-throughput genotyping platforms to build groups of genes with related expression patterns (also known as coexpressed genes). The algorithms we have used are hierarchical algorithms that find successive clusters using previously established clusters and create a hierarchy of clusters which may be represented by a hierarchical clustering dendrogram. The method builds the hierarchy from the individual elements by progressively merging clusters. In particular, hierarchical cluster analysis of genes showed the differential expression of genes in HepG2 cells respect to human hepatocytes used as healthy controls. 2646 genes were significantly down-regulated in HepG2 cells respect to the hepatocytes whereas a further 3586 genes were significantly up-regulated. Moreover, information on the biological functions of the genes that were significantly regulated was obtained by a pathway analysis. Pathways related to these genes were extracted from KEGG (Kyoto Encyclopedia of Genes and Genomes) (Ogata et al 1999), Pathway Interaction Database, and network for CXCL12 in Hepatocellular carcinoma is derived from the HCCnet (Hepatocellular carcinoma network database) (Bing et al, 2010), which contains around 37811 protein-protein interactions from 13 individual datasets having 894 HCC samples containing 30.5%, 44.7 % and 18.7% of HCV infected, HBV infected and unknown factors respectively. Indeed a network of transcription factors that are extracted from microarray data and interact with EGFR gene is constructed by Cytoscape.

Amongst the significantly up-regulated and down-regulated genes several chemokines and some transcription factors (CCL20, CCR6, CX3CL1, Grb2, p53, VCAN, C-MYC, CXCL12, SDC4 and Cyclin D1) were found. CCR6, being the receptor for the chemokine CCL20, is expressed on inactivated memory T-cells, on some dendritic cells and also on Th17 cells. Some studies suggest the involvement of the CCL20/CCR6 system in the carcinogenesis and progression of human HCC (Rubie et al., 2006). CCR6 is implicated in the Chemokine signaling pathway and cytokine–cytokine receptor interaction as obtained from Kegg

(Kyoto encyclopedia of genes) (Ogata et al 1999). In particular, CCL20 interacts with VCAN gene (Fig. 3) which encodes for the Versican protein that plays a role in angiogenesis, inflammation. This protein prevents the growth of cancerous tumors and regulates the activity of several growth factors, which control a diverse range of processes important for cell growth. Moreover elevated levels of Versican have been reported in most malignancies, including brain tumors, melanomas, lymphomas and breast cancers, prostate, colon, lung, pancreas, endometrium, and ovary (Miranda et al., 2011; Kusumoto et al., 2010). CCR6 and CCL20 are known to interact with Versican in the HCC network. This protein was found to be upregulated in HCC patients correlated to Hepatitis B Virus and was indicated as a possible candidate for mediating tumor progression and proliferation in liver and more importantly visual impairment (Paraneoplastic syndrome) associated with HCC patients correlated to HBV.

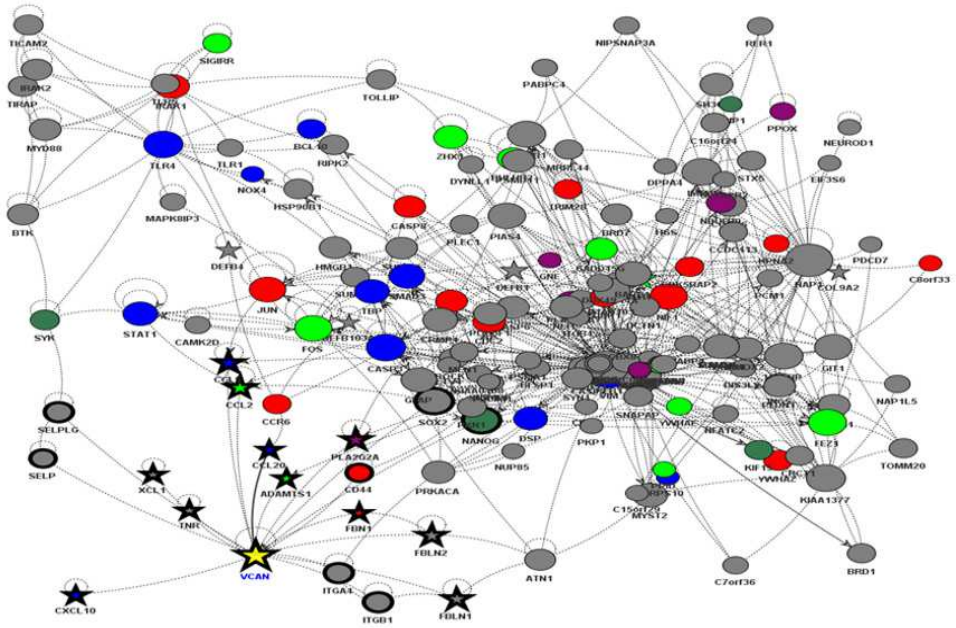

Fig. 3. VCAN Network where the genes are shown by circle and proteins by stars. Different colors: red, up-regulated one; light green, down-regulated one; blue, bidirection-regulated one; grey, not high confident HCC-related one; dark green, genes with selected function info; purple, genes with selected pathway info.

The CXCR4/CXCL12 axis is up-regulated in HCC and participates in HCC cell proliferation. Upon interaction with SDC4 (Syndecan 4), being a heparan sulfate proteoglycans, these proteins function as key regulators of cell signaling via their interactions with multiple growth and angiogenic factors, and promote an aggressive tumor phenotype (Sanderson et al, 2010). CXCL12/SDC4 makes a complex with O Phospho L Tyrosine and upon complex formation it can be hypothesized that it provides stability to p53 protein avoiding cancerous sitations as shown in various cancer cell lines like cervical cancer and lung cancer Renal and

Lung cancer (Sanderson et al, 2010). CXCL12 is found to trans-activating Epidermal growth factor receptor (EGFR) (Porcile,C et al ,2005), that is considered as an important signaling hub where different proliferative and survival signals converge. It is highly evident that EGFR has most important roles to play for controlling signaling cascades from extracellular regions. In particular, the interaction network of EGFR with transcription factors can provide much needed insights to multi factor governing HCC. There were found numerous up- and down- regulated transcription factors interacting with EGFR from microarray data (Fig. 4) and they can produce actions in invasion and metastatis state of the cells and induce simulateounously many biological processes to prevent metastatis, invasion and damage to liver cells.

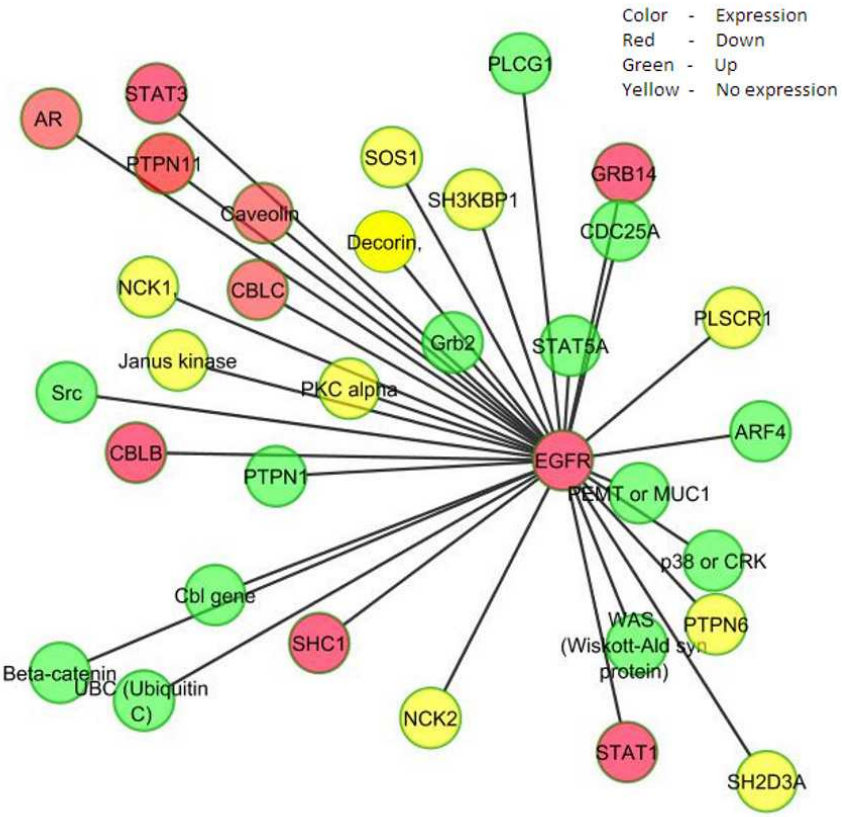

Fig. 4. Interactions of EGFR with de-regulated genes of our microarray data. The down-expressed genes are reported in red, those up-regulated in green and in yellow those no expressed.

Moreover, between the important factors that are governing HCC there is also CX3CL1 (Fractalkine), being a chemokine with both chemoattractant and cell-adhesive functions. Under specific inflammatory conditions, it could regulate the attractions of CX3CR1

bearing cells to tumor site either providing antitumor responses or either pathogenic angiogenesis (Deleterious effects) (Turner et al, 2010). When CX3CL1 is expressed in the tumor, it appears to recruit cytotoxic T cells and NK cells to the tumor site and its expression level is found to correlate with the density of Tumor Infiltrating Lymphocytes (TILs) in some cancers (Ohta et al ,2005).

In addition to these chemokines, Grb2 is also one of the most important upregulated proteins in HCC that was found to be functioning in number of pathways involved in cancer. In particular, Grb2 recruits SOS (exchange protein) for the activating RAS that operates as a molecular switch between MEK and ERK (MAPK) which in nucleus acts on numerous important transcriptions factors like STAT 3 and the expression of STAT3 regulates genes including BCL-x1, CYCLIN D1 and c-MYC which involve in cell apoptosis and cell cycle progression (Sun et al.,2008). In IL6 mediated signaling events, GRB2 interacts with some proteins like FOS and JUN, that are both down regulated in HCC and importantly transcriptional activity of JUN is attenuated and sometimes antagonized by JUNB. This activation takes place in chemically induced murine liver tumours and HCCs of humans, suggesting oncogenic function for this gene in liver tumors of mammals with HSP70 that exhibits regulatory functions of c-JUN, ERK and the JNK pathway, thus inhibiting cell apoptosis (Lee et al., 2005). Moreover, Grb2 plays a specific role in EGF-stimulated EGFR internalization (e.g. receptor sorting, vesicle budding/pinching or vesicle transport (Yamazaki et al., 2002).

From KEGG, other two pathways are found to be deregulated in HCC metastasis, i.e. P53 and MAPK pathways. TP53 plays an important role on regulation of apoptosis and cell cycle arrest and external environment factors or agents are implicated in the development of HCC in correlation with P53, including nutrition, diabetes, oral infection, oral contraceptive, alcohol consumption and some trace elements such as Selenium (Irmak et al., 2003; Wei et al., 2001). A second pathway, which is deregulated in metastatis, is MAPK pathway that is considered to control the most of the activities related to HCC condition by activating around 90 transcription factors although tyrosine kinase inhibitor Sorafenib is used as potential inhibitor of MAPK pathway by inhibiting RAF in HCC and Renal carcinoma (Cabrera et al., 2011).

6. Evaluation of cytokines in HCC patients with HCV-related cirrhosis

The serum levels of 50 different cytokines, chemokines and growth factors were evaluated in patients affected by HCC with chronic HCV-related hepatitis and liver cirrhosis using multiplex biometric ELISA-based immunoassay (Capone et al., 2010). The HCC patients showed a different secretion profile of these proteins compared to healthy controls. Greater amounts of IL-1α, IL-3, IL-12p40, IL-6, IL-8, IL-10, CCL27, CXCL10, CXCL1, IFN-α2, M-CSF, GM-CSF, CXCL9, β-NGF, SCF, SCGF-β, CXCL12, TNF-β were secreted by the HCC patients. No correlation was observed between serum levels and patients age/gender or between patients with a solitary tumour and those with multiple tumours. In particular, the attention was focused only on the proinflammatory molecules (IL-1α, IL-6, IL-8, IL-12p40, GM-CSF, CCL27, CXCL1, CXCL9, CXCL10, CXCL12, β-NGF) that were found to be significantly increased in HCC patients compared to healthy controls. The significantly increased serum levels of IL-6 and IL-8 found in HCC patients agreed with data reported in other studies (Ataseven et al., 2006; Burger et al., 2006). In particular, IL-8 levels measured in HCC patients were found to be increased, and correlated significantly with large tumor size (> 5

cm) suggesting that IL-8 may be involved in disease progression and might prove to be both a useful marker of tumor invasiveness and an independent prognostic factor for HCC patients (Burger et al., 2006, Capone et al., 2010).

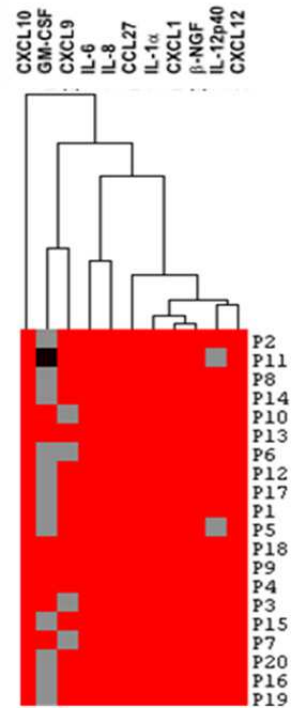

Fig. 5. Protein hierarchy assessed by a computational clustering analysis. More specifically, the length of branches indicates, in inverse proportion, the similarity of protein concentrations, and the scale of protein intensity is indicated by the different colors: over-expressed cytokines, chemokines and growth factors in red, lower values in grey, and values equal to zero in black.

Since IL-8 and IL-6 concentrations correlated significantly with large tumor size (p-value < 0.05 and R > 0.83), this confirmed the clinical significance of IL-6 as a prognostic factor of cancer and, in particular, its association with the development of HCC (Łukaszewicz et al., 2007; Wong et al., 2009; Nakagawa et al., 2009). Indeed, CXCL10 levels correlated both with any tumour size and with transaminase levels suggesting that it could be used as marker of liver inflammation status and cancer progression. CXCL12 is known to play a role both in pathogenesis by promoting tumor growth and malignancy, and in the HCC metastatic network by recruiting endothelial cell tumor progenitors (Liu et al., 2008; Burns et al., 2006; Kryczek et al., 2007). Recently, some papers have suggested that β-NGF was involved in cancer growth and metastasis and was detected in diseased liver tissues; in fact this protein has been suggested to be involved in chronic inflammation leading to cancer (Rasi et al., 2007). The correlation evaluation between the concentrations of over-expressed pro-inflammatory molecules measured in HCC patients showed that β-NGF correlated with IL-

1α, IL-12p40, CCL27, CXCL1 and CXCL12. This was confirmed by the related computational clustering analysis which shows that the molecules cluster in two groups, as demonstrated by branches joining them. In particular, β-NGF was grouped with the proteins indicated above (Fig. 5). Therefore, it is possible to suggest that a panel composed of β-NGF and these five proteins may be useful for diagnostic/prognostic purposes.
In conclusion, this approach showed that some pro-inflammatory molecules were significantly up-regulated in these patients, and highlighted the complexity of the cytokine network in this disease. Moreover, this suggests the need to monitor these proteins in order to define a profile that could characterize patients with HCC or to help identify useful markers. In fact, this could lead to better definition of the disease state, and to an increased understanding of the relationships between chronic inflammation and cancer (Capone et al., 2010).

7. Evaluation of cytokines in patients with chronic HCV or with HCV-related cirrhosis

The serum concentrations of a panel of 30 cytokines, chemokines and growth factors were evaluated in patients with chronic inflammation (HC) and liver cirrhosis (LC), and in healthy donors by multiplex biometric ELISA-based assays (Costantini et al., 2010a). The molecules that showed different serum levels in patients respect to healthy controls are reported in Table 1.

	HC vs controls	LC vs controls
IL-1a	0.0196*	0.0077**
IL-1b	<0.0001***	<0.0001***
IL-2R	0.0355*	0.0053**
IL-6	0.0032**	0.0024**
IL-8	0.0004***	0.0001***
CXCL1	0.0076**	0.0034**
CXCL9	0.0004***	0.0002***
CXCL10	0.0015**	0.0003***
CXCL12	0.0364*	0.0443*
MIF	0.04*	0.0033**
b-NGF	0.0008***	0.0002***
HGF		0.0028**

Table 1. P values obtained for all significant molecules in HC and LC patients respect to controls using the nonparametric Mann-Whitney U test.

Greater amounts of IL-1α, IL-1β, IL-2R, IL-6, IL-8, CXCL1, CXCL9, CXCL10, CXCL12, MIF, and β-NGF were secreted by both HC and LC patients.
In particular, in the chronic inflammation and liver cirrhosis patients the same proteins were increased and the only difference was related to HGF being resulted significant and up-regulated only in the patients with liver cirrhosis and not in those with chronic

inflammation. In particular, HGF is a multifunctional growth factor that regulates growth and cell motility, exerts mitogenic effects on hepatocytes and epithelial cells and plays diverse roles in organ development, tissue regeneration, and tumor progression (Gentile et al., 2008). Moreover, it is implicated with IL-6, IL-8 and IL-1 in the hepatic stellate cell activation pathway.

However, numerous reports have examined the relationship between HGF and either the facilitation or suppression of HCC occurrence and have suggested that this growth factor could be used as index of cellular growth and of HCC development in liver cirrhosis patients (Yagamamim et al., 2002). In fact, it is interesting that the amount of this molecule was significantly different in liver cirrhosis patients in respect to both healthy controls and chronic inflammation patients and that its concentration in HCC patients was higher than in liver cirrhosis patients. This means that HGF increased in the progression of chronic inflammation leading to liver cirrhosis and cancer and can be used for predicting the occurrence of HCC in chronic HCV-related liver diseases (Costantini et al., 2010a).

7.1 Chronic inflammation versus liver cirrhosis patients

Since IL-1α, IL-1β, IL-2R, IL-6, IL-8, CXCL1, CXCL9, CXCL10, CXCL12, MIF, and β-NGF were increased in both HC and LC patients in respect to healthy control subjects, their mean concentrations were compared by t-test. Fig. 6 shows that the concentrations of all the proteins and, in particular, IL-8, CXCL9 and β -NGF were higher (with $p<0.05$) in patients with liver cirrhosis than in those with chronic inflammation. Afterwards, comparing the serum levels of all cytokines, chemokines and growth factors in HC and LC patients respect to those in HCC patients tested in our recent paper (Capone et al.,2010) is resulted that the mean concentrations of all molecules resulted higher in HCC patients than in those with liver cirrhosis. This indicates that the expression of these pro-inflammatory molecules tends to increase in the chronic inflammation progression leading to liver cirrhosis and HCC and, thus, their evaluation could be used for prognostic studies. The serum levels of statistically significant cytokines, chemokines and growth factors in the HC and LC patients were correlated with clinical data by using the Pearson correlation coefficient. In chronic inflammation patients IL-1α, IL-2R, MIF and β-NGF showed a significant correlation with a positive correlation coefficient between them and with the transaminase values, that were higher in these patients than in healthy controls. Therefore these proteins can be considered as index of immune activation. In particular, these results agreed with literature data reporting that IL-1 and IL-2R participate in the progression from liver injury to fibrosis (Zekri et al., 2010) and that β-NGF is involved in liver cancer growth and metastasis and can be used as an index of chronic infection leading to LC and HCC (Gieling et al., 2009). Indeed, this work suggested for the first time a role of MIF in HCV-related chronic inflammation patients because an increased serum MIF was reported only in HBV patients (Kimura et al., 2006). Moreover, CXCL1, CXCL9, CXCL10 and HFG in liver cirrhosis patients showed a significant correlation and, in details, a positive correlation coefficient between them and a negative correlation coefficient with the albumin values, that were lower in these patients respect to controls. Concerning that HGF resulted the only molecule that was statistically different between HC and LC patients, these data suggested that the four proteins could be useful for diagnostic/prognostic purposes.

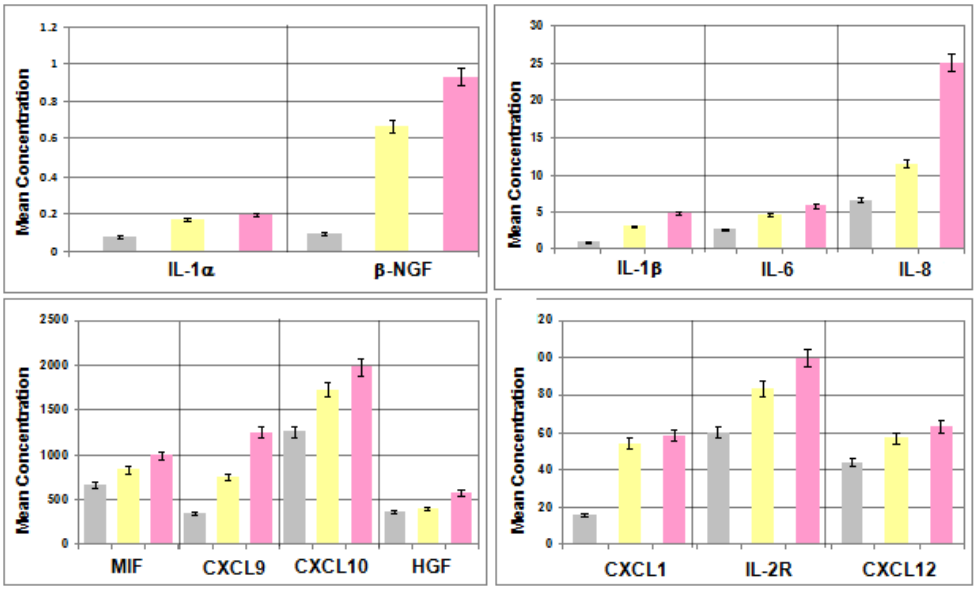

Fig. 6. Mean concentrations of significant cytokines, chemokines and growth factors in healthy control subjects (light grey) and in chronic inflammation (yellow) and liver cirrhosis (pink) patients.

7.2 Chronic inflammation patients with different fibrosis stages

After the classification of their fibrosis stage of chronic inflammation by F Ishak index (Costantini et al., 2010a), the patients were divided in three different subgroups corresponding to F2, F3 and F4 stages and their mean concentrations of significant molecules in three groups compared by t-test. No relevant difference was found between chronic inflammation patients with F3 and F4 fibrosis grade because they corresponded to two already advanced fibrosis stages. Comparing F2 and F4 patients the concentrations of IL-2R, IL-6, IL-8, CXCL9, CXCL10, CXCL12 and MIF were found statistically higher (with $p<0.05$) in chronic inflammation patients with F4 fibrosis grade in respect to F2 fibrosis grade (Costantini et al., 2010a). These data agreed with a recent paper reporting that CXCL9 and CXCL10 were significantly elevated in patients with advanced fibrosis (Zeremski et al. 2009).

In conclusion these results suggested that i) IL-2R, IL-6, IL-8, CXCL9, CXCL10, CXCL12 and MIF could be markers of the progression of chronic hepatitis C leading to liver cirrhosis by increasing fibrosis and ii) HGF, being over-expressed only in liver cirrhosis patients, could be index of fibrosis progression versus liver cirrhosis.

However this work indicated the need of cytokinome data mining system for a predictive medicine, and suggested the utility to integrate all the cytokine data in a network and to make drug design studies on the chemokines resulted significant in the progression from chronic inflammation to HCC.

8. The need of cytokinome data mining system for a predictive medicine

The progressive increase in electronically stored clinical data is opening the possibility of carrying out large-scale studies aimed to discover correlations between new research data and related diseases. For these reasons, many relational databases have implemented data mining techniques (Harrison, 2008) that have been described as the 'extraction of implicit, previously unknown and potentially useful information', such as associations and correlations between data elements from large repositories of data (Lee & Siau, 2001). However, the scientific community needs clinical laboratory databases to collect medical data related to diseases progression and therapy response. In the last years, particular attention has been focused on the protein class comprising cytokines, chemokines and growth factors, because they play a crucial role in promoting angiogenesis, metastasis and subversion of adaptive immunity. Since the control of cytokine production is highly complex and multifactorial, their effects are mediated through multiple regulatory networks. The intricate complexity of these networks clearly conceals the role that a single cytokine may play in the pathogenesis of the disease. Therefore, it is more informative to investigate the immunopathogenesis of a disease process by analyzing a multiple panel of cytokines (Costantini et al., 2009). Utilizing a bead-based broad-spectrum multiplex immunoassay, it is possible not only to evaluate the serum levels of those cytokines ensemble that effectively correlate with the progression of the disease activity but also to define the immunomodulatory effects of a therapy even after months of treatment (Sato et al., 2009; Ozturk et al., 2009; Capone et al., 2010). This indicates that the definition and evaluation of a human cytokinome represents an important future tool to analyze the interaction network of cytokines both in healthy individuals and in patients affected by different diseases. In fact, it will permit one to understand and investigate how the regression of a chronic inflammation process, by acting on the cellular populations of cytokines, can block the progression of a cancer and, therefore, it can be a useful prognostic and diagnostic tool for clinicians.

For these reasons, a portal with user-friendly interfaces, which can be used both by physicians and researchers not only to collect and to correlate clinical data and serum levels of cytokines but also to know quickly what cytokines, chemokines or growth factors are significant in the progression state of a given disease, represents an important and useful tool for clinical prognosis and therapy studies.

Recently it has been developed a software named CDMS (Clinical Data Mining Software) and accessible at the URL: http://www.cro-m.eu/CDMS/ to collect clinical data and serum levels of many cytokines, chemokines and growth factors evaluated on healthy subjects and patients affected by different diseases (i.e. chronic hepatitis C and HCC) using multiplex immunoassays (Evangelista et al., 2010). Moreover, some statistical tools were implemented to correlate significatively clinical and experimental data and to quickly compare standardized cytokinome profile of a patient against a whole data bank that collects cytokinome data from some different diseases. CDMS allows certified users to access some of its services on the basis of their privileges. In detail, physicians and researchers can access the patient administration and statistical analysis sections, and all other authorized figures can access only statistical analysis section. In the patient administration section, there are case histories of patients with information related to their diagnosis, biological analyses as well as clinical data, and evaluations of 50 cytokine concentrations. Moreover, for the same patients, it is possible to insert the cytokine profiles evaluated at different times to compare

and evaluate results at different stages of the disease. In the statistical analysis section, the user can select the disease, filter the patients on the basis of gender, age and experiment date and select the most appropriate tool to perform the statistical analysis. In particular, we have implemented: (i) median, mean, variance, standard deviation, min and max values for the selected protein; (ii) t-test value related to the comparison between cytokine concentrations in control group and patients; (iii) Pearson correlation between different cytokines with related graph; (iv) Pearson correlation between each cytokine and some clinical data (i.e. tumor size) with related graph. CDMS represents the first 'user-friendly' tool that can be used by researchers as well as physicians and clinicians to significatively correlate clinical data and cytokine profiles and to identify what cytokines can be significant for the examined disease at a given time. Using its available statistical tools, it has been possible to identify the cyto-chemokines pattern involved in the chronic inflammation processes versus HCC and to verify that IL-8 correlated significantly with large tumor size (>5 cm), and it can be used both as a useful marker of tumor invasiveness and as an independent prognostic factor for HCC patients (Capone et al., 2010; Evangelista et al. 2010). Therefore, this tool can be a useful support to develop a reliable predictive medicine and to improve or discover new predictive relationships among data groups.

9. The need for structural studies of cytokine/receptor complex: The example of CXCL9, CXCL10 and CXCL11 chemokines and their membrane receptor CXCR3

The data obtained on sera of patients with chronic inflammation (HC), liver cirrhosis (LC) and HCC suggested the utility to make drug design studies on three CXCL9, CXCL10 and CXCL11 chemokines for obtaining molecules able to block the progression of fibrotic damage in chronic inflammation patients leading to liver cirrhosis and, then, to HCC (Costantini et al., 2010a).

CXCL9, CXCL10 and CXCL11 are members of a family of small (8–10 kDa) proteins, the chemokines (or chemoattractant cytokines). They play a key role in immune and inflammatory responses by promoting recruitment and activation of different subpopulations of leukocytes, hence they have important proinflammatory and immune modulatory functions (Booth et al., 2002). CXCL9 as well as do CXCL10 and CXCL11 binds and activates the same receptor CXCR3 (chemokine (C-X-C motif) receptor 3) (Booth et al., 2004).

CXCR3 is mainly expressed on activated T and Natural Killer (NK) cells (Zeremski et al., 2007). While CXCL11, CXCL10, and CXCL9 are agonists for CXCR3, they can also act as antagonists for CCR3 (Loetscher et al. 2001). Tumor cells aberrantly express chemokines and/or chemokine receptors, and the interaction of chemokine ligand-receptor pairs is increasingly implicated as a mediator of tumor growth and metastasis. In particular, CXCR3 has now been identified in a variety of malignant cells, including melanoma, breast and prostate carcinomas, neuroblastoma, and a subset of B cell lymphomas (Colvin et al., 2004). CXCL9 and CXCL10 may promote the recruitment of lymphocytes to HCC and released from the HCC cells may induce lymphocyte infiltration. Ruehlmann et al. (2001) suggested that the expression of CXCL9 and CXCL10 might lead to lymphocytic infiltration into HCC, and gene therapy with these CXC chemokines may be effective for patients with HCC. Hrnce, during the past few years, several studies have demonstrated a pathogenenetic role of CXCR3 and its ligands in human inflammatory diseases suggesting the involvement of

various segments of their sequences. Therefore, the blockade of CXCR3 interactions with its ligands in vivo has been suggested as a possible therapeutic goal for the treatment of these disorders (Xanthou et al. 2003). Recently the three-dimensional structure of CXCL9 and CXCR3 has been simulated (Trotta et al., 2009). Successively, also the CXCL9/CXCR3 complex (Fig. 7) has been modelled in comparison to CXCL10/CXCR3 and CXCL11/CXCR3 complexes in order to evaluate in details the interaction residues involved in the formation of the complexes and their properties as important structural features to be used for drug design (Trotta et al., 2009).

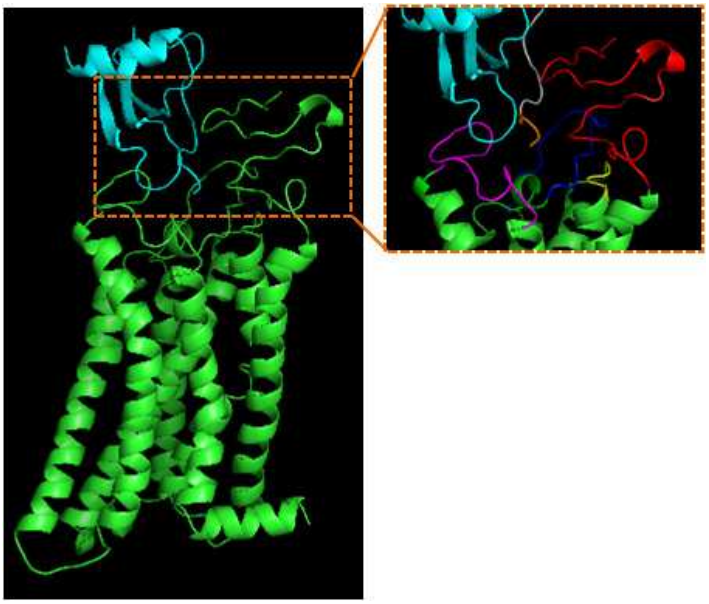

Fig. 7. 3D model of CXCL9/CXCR3 complex where CXCR3 is reported with green ribbon but CXCL9 with cyan ribbon. In details, the loops of the interaction regions are evidenced (i.e. N-terminal, loop1, loop 2 and loop3 of the receptor are shown in red, yellow, blue and magenta, respectively, and N-terminal and N-loop of the chemokine in orange and grey, respectively).

Three chemokines resulted always to interact with their receptor by N-terminal region and N-loop but the receptor by N-terminal region and three extracellular loops according to precedent studies (Xanthou et al. 2003). Moreover the analysis of three complexes showed that the N-loop of all three chemokines was essential for binding the N-terminal region of CXCR3 in agreement to Clark-Lewis et al. (2003) whereas the loop 1 of CXCR3 was essential to bind only CXCL11 and CXCL10 as well as indicated by Xanthou et al. 2003. The analysis of the physical-chemical properties of residues present in these regions in CXCR3 highlighted that: i) N-terminal, loop1 and loop 2 contained some aromatic residues (Phe, Tyr and Trp); ii) N-terminal presented three negatively charged residues (3 Glu), loop2 one (Asp) but loop 3 three (2 Asp and 1 Glu) and iii) both loop 2 and loop 3 had two positively charged residues (2 Arg).These data suggested that the predominant interaction between CXCR3 and its ligands was on electrostatic basis and was favored also from the presence of

positively charged residues located in N-terminal region of three chemokines (i.e. three in CXCL9 and CXCL11 and two in CXCL10). Moreover, the presence of aromatic residues stabilized mainly the interaction between CXCR3 and CXCL11, having two Phe residues in N-terminal and might play an important role to favour the stacking interactions with putative drugs and organic compounds.
Therefore the study of the structural basis of the CXCR3 receptor-ligand system through the modeling of three complexes CXCL9/CXCR3, CXCL10/CXCR3, and CXCL11/CXCR3 has evidenced the interaction regions between three chemokines and CXCR3 (Trotta et al., 2009). The related analysis of the physico-chemical properties of residues in these regions suggested that the predominant interaction between CXCR3 and its ligands was on electrostatic basis and favored by the presence of positively charged residues located in the N-terminal region of the three chemokines. The comparison of the three complexes showed that CXCR3 had the highest affinity for CXCL11 in terms of binding energy and higher number of H-bonds, of salt bridges and of interaction residues (Trotta et al., 2009). Since the in silico modelling provided a time- and cost-effective tool for the screening of molecules as well as for designing of novel molecules of desired activity, it was possible to focus the attention on CXCL11. Therefore, in order to develop putative antagonists to CXCR3, a peptide, derived from the N-terminal region of CXCL11, has been synthesized. Preliminary results of this study, taken as a whole, indicated that this peptide may be regarded as a small molecule that, opportunely modified, could represent a good model for an antagonist to CXCR3. Hence, further studies are currently underway to design analogs of this peptide to optimize its physico-chemical properties and to improve the electrostatic and stacking interactions with CXCR3 for novel therapeutic approaches.

10. Conclusion

Over the past several years, there has been a renaissance of research into connection between inflammation and cancer. The inflammation can play a role in tumor suppression by stimulating an antitumor immune response, but more often, under certain conditions, it appears to stimulate tumor development (Mantovani et al., 2008). The intensity and nature of the inflammation could explain this apparent contradiction. In fact, the inflammation may become chronic when the inflammatory stimulus persists. However, it has been suggested that inflammation associated with cancer is similar to that seen with chronic inflammation, which includes the production of growth and angiogenic factors that stimulate tissue repair, factors that can also promote cancer-cell survival, implantation, and growth (Allavena et al., 2008). Thus immune response can promote anticancer effects or carcinogenesis and tumor growth (Mantovani et al., 2008). Cytokines are among molecules that play an important role in the evolution of these processes. In fact, they are proteins that are expressed before and during the inflammatory process and play a key role at the various disease levels so that they can be considered as specific markers of cancer and of its specific evolutive steps (Capone et al., 2010; Costantini et al., 2010a).
The studying model chosen in this chapter is the hepatocellular carcinoma (HCC) that represents a major health problem worldwide being the fifth most common malignancy in men and the eighth in women and the third most common cause of cancer-related death in the world. Indeed its incidence is increasing dramatically, with marked variations among geographic regions, racial and ethnic groups, relatively to the exposure documented environmental risk factors (Castello et al., 2010a, 2010b). In particular, Southern Italy has the

highest rates of HCC in Europe (Fusco et al., 2008). HCC derives from a long clinical history in patients with HCV or HBV infection. In fact, about 80% of newly infected patients develop chronic infection; an estimated 10% to 20% will develop cirrhosis and 1% to 5% advance to end-stage liver cancer (HCC) over a period of 20 to 30 years (Fig. 1).

Recently the serum levels of many cytokines have been evaluated by a broad spectrum bead-based multiplex immunoassay both in patients with chronic HCV or with HCV-related cirrhosis and in patients with HCC patients with HCV-related cirrhosis. These studies have evidenced that some interleukins and chemokines (Fig. 5 and 6) are putative markers of the progression of chronic hepatitis C leading to liver cirrhosis by increasing fibrosis and can be used as templates for designing new drugs able to block the progression of the inflammatory processes (Capone et al., 2010; Costantini et al., 2010a).

However, all the data related to the cytokine evaluations should be modeled computationally by using graphs or networks connecting the various data groups in terms of dynamic probabilistic maps of metabolic and/or physiological activities and/or pathogenetic pathways. In fact only in this way it is possible to define the human cytokinome that can be an useful tool to analyze the interaction network of cytokines both in healthy individuals and in patients affected from HCC (Costantini et al., 2010b). Therefore, CDMS represents the first 'user-friendly' tool that can be used by researchers as well as physicians and clinicians to significatively correlate clinical data and cytokine profiles and to identify what cytokines can be significant for the examined disease at a given time (Evangelista et al., 2010). However further studies will regard the opening of the data sets to other diseases and the implementation of other statistical tools and classification methods to improve or to discover new predictive relationships among data groups.

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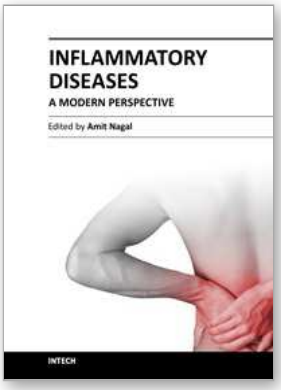

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"Inflammatory Diseases - A Modern Perspective" represents an extended and thoroughly revised collection of papers on inflammation. This book explores a wide range of topics relevant to inflammation and inflammatory diseases while its main objective is to help in understanding the molecular mechanism and a concrete review of inflammation. One of the interesting things about this book is its diversity in topics which include pharmacology, medicine, rational drug design, microbiology and biochemistry. Each topic focuses on inflammation and its related disease thus giving a unique platform which integrates all the useful information regarding inflammation.

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